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Technical and scientific translation – German and French into English

Declaration

I, Nicholas Hartmann, translator, having an office at 828 N. Broadway, Suite 506, Milwaukee, WI, 53202, declare that I am well acquainted with the English and German languages and that the appended document is a true and faithful translation of:

International patent application PCT/DE99/03527 (WO 00/31576) entitled

"Verfahren zur Einstellung der Systemparameter eines Laserscanmikroskops"

All statements made herein are to my own knowledge true, and all statements made on information and belief are believed to be true; and further, these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the document.

Date May 3, 2001 ..... Nicholas Hartmann

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Method for adjusting the system parameters of a laser scanning microscope

The invention concerns a method for setting the system parameters of a preferably confocal laser scanning microscope, setting of the system parameters being accomplished by way of a control computer.

The invention refers to the field of laser scanning microscopy, in particular to the field of confocal laser scanning microscopy. Laser scanning microscopes have been known from practical use for years. Purely by way of example, the reader is referred in this context to DE 196 54 211 A1. Confocal laser scanning microscopes demand of the user sufficient knowledge about the operation of such a laser scanning microscope, specifically in order to set the mutually dependent and often also contradictory or mutually exclusive system parameters. These include the pinhole diameter, the high voltage of the photomultiplier (PMT), the laser output, etc. For optimum setting of the system parameters, in particular in consideration of specimen-specific properties, the user must draw upon his or her experience with such laser scanning microscopes. Until now, however, it has been almost impossible for a user to achieve optimum imaging results without extensive relevant experience.

Particularly in terms of quantitative confocal laser scanning microscopy, an assessment of the acquired image data in terms of data quality is an important criterion. The quality feature of the acquired data can be, for example, the signal-to-noise ratio or the resolution that is achieved. An optimal data acquisition strategy is therefore a prerequisite for successful quantitative confocal laser scanning microscopy on a broadly applied basis.

Because of the aforementioned complexity in the setting of confocal laser scanning microscopes, many users do not optimally set the operating or system parameters of laser scanning microscopes. A lack of knowledge of the sometimes very complex correlations among different optical and electronic boundary parameters of a confocal laser scanning microscope is the principal reason for hitherto inadequate operation. But if a laser scanning microscope of this kind is not optimally set, an image can be acquired only with reduced image quality

or with much too long a setting procedure prior to actual image acquisition. Too long a setting phase prior to actual image acquisition, however, reduces the efficiency of such a microscope, and usually results in excessive wear on the laser light source and/or the light-guiding fibers impinged upon by the laser light, and possibly in damage to the specimen.

The laser scanning microscopes heretofore known from practical use are also problematic, in particular, when new users are being trained, since instruction and assistance from experienced users are always necessary. In all cases, it has hitherto been difficult to teach oneself to use a laser scanning microscope optimally. Indeed, with existing laser scanning microscopes an extremely long training phase, with the assistance of experienced users, is absolutely necessary.

A further problem arising from existing practice is the fact that many fluorescent specimens bleach out with very long setting phases. But since long setting phases in most cases cannot be ruled out, the use of the laser scanning microscopes hitherto known from practical use is limited, especially in the case of biological specimens, and thus problematic. A very considerable reduction in the time required for optimum setting is thus fundamentally desirable.

In light of the aforementioned problems, it is the object of the present invention to describe a method for setting the system parameters of a preferably confocal laser scanning microscope, setting of the system parameters being accomplished by way of a control computer. This method is intended to make possible reliable and also reproducible setting of the laser scanning microscope, specifically in consideration of predefinable system and specimen parameters.

The method according to the present invention achieves the aforesaid object by way of the features of Claim 1. According to the latter, the method for setting the system parameters of a preferably confocal laser scanning microscope is characterized by an interactive user interface, such that upon

input of at least one specimen parameter and/or at least one optionally selectable system parameter, settings for the remaining system parameters are proposed to the user and/or the remaining system parameters are set automatically.

What has been recognized according to the present invention is that a reduction in the time necessary for optimum setting is possible, in reasonable and reproducible fashion, only if an interactive user interface based on the physical correlations and the formulas hereinafter is accomplished. In other words, the control computer has available a corresponding software program which interactively generates a user interface. Upon input of at least one specimen parameter and/or optionally of a selectable system parameter, settings for the remaining system parameters are proposed to the user. Following selection, or (also selectably) automatically, the remaining system parameters are set on the basis of the software's proposal. What is essential is that a quasi-optimal setting of the system parameters is always interactively proposed. Depending on the selection of an individual system parameter or several system parameters, the remaining system parameters are adapted to the selection that is made, and additional optimization is performed in the context of the definition or definitions. The same applies to the specimen parameters.

Advantageously, the method according to the present invention includes the possibility that upon input of at least one specimen parameter and/or of an optionally selectable system parameter and/or of at least one definable problem regarding image acquisition and/or regarding the specimen that is to be imaged, optimization paths for system setting and/or imaging strategies are proposed. The user interface thus comprises a quasi-intelligent system or a corresponding database which, after definition of individual specimen/system parameters or after definition of a specific problem, proposes optimization paths for system setting or imaging strategies. The user can then, on the basis of his or her definitions, select an imaging strategy that is optimal for his or her needs.

The predefinable system parameters of a selected system setting or imaging strategy can - selectably - be set automatically via the user interface, preferably after a confirmation to be entered by the user. A security query can be provided in this context.

Numerous specimen/system parameters can be interactively (pre)selected. These include, for example, the specimen dimension to be imaged, the specimen region to be imaged, the number of optical steps in or through a specimen, the specimen property to be imaged, detection method, etc. Suitable detection methods can involve the use of the fluorescence method and the reflection method. Note in this connection that definition of the aforesaid parameters can be accomplished by unrestricted input or by selection from a predefined menu that optionally can be expanded by the user.

It is additionally possible for the parameters regarding the apparatus to be interactively selectable or definable. For example, the use of a suitable objective having the highest possible numerical aperture to achieve maximum resolution could be proposed, specifically on the basis of previously selected system/specimen parameters. The maximum resolution achievable with the selected objective could also be reported interactively. On the basis of previously selected or ascertained (and optionally previously set) system parameters, the resolution presently achievable could be reported, specifically so the user can check that said resolution is in fact sufficient on the basis of the predefined system parameters. The user interface could thus propose alternative settings, specifically in order to achieve higher resolution.

It is also conceivable for the number of pixels per image plane to be proposed interactively.

The specimen property to be entered or selected interactively serves to determine the optimum irradiation intensity, which is also proposed interactively for setting. The optimum irradiation intensity or laser output is also proposed interactively, again on the basis of definable

system/specimen data. It is furthermore conceivable for the optimum irradiation intensity or laser output to be set automatically, optionally after authorization by the user.

For setting the detection pinhole diameter, an optimal value at which the image acquisition resolution is maximal, while the image acquisition signal-to-noise ratio is still usable, is proposed interactively. It is also conceivable, in order to set the detection pinhole diameter, for an optimum value at which the image acquisition signal-to-noise ratio is maximal, while the image acquisition resolution is still usable, to be proposed interactively.

As already mentioned previously, the user interface provided for in accordance with the invention offers very considerable advantages and simplifications. This is also true, in additionally advantageous fashion, for the fact that upon definition or modification of at least one system parameter, all those system parameters that are influenced by the definition or modification are reported interactively. By means of the interactive user interface, a report can be given as to how, on the basis of the definition or modification of parameters, an image acquisition can be performed with the best possible image quality. The user can thus select a variety of optimization paths, specifically on the basis of defined specimen/system parameters and on the basis of modified specimen/system parameters.

In very particularly advantageous fashion, especially with regard to particular imaging techniques or specific applications, it is very particularly advantageous if at least one criterion that is important for imaging or for application can be defined for the optimization thereof. Based on this definition, in the context of the user interface the further system parameters are then proposed interactively and/or (possibly following a query and a user-provided confirmation) automatically set. The predefined criterion can be, for example, the signal-to-noise ratio that is to be achieved. The definition of other criteria is also possible, in which context an adaptation of the remaining system parameters (thereupon) takes place.

By means of the user interface, assistance or solutions for predefined problem situations could be offered interactively. The problem situations could be a variety of problem cases, for example the problem case in which the specimen (in the case of fluorescence specimens) bleaches excessively, in which the image data are too "noisy," in which the measurement time is too long, or in which the image acquisition resolution is too low. In accordance with this problem situation, assistance or solutions which are selectable by the user on the basis of previously set system parameters are offered interactively. Optimization is (interactively) possible on the basis of the interactively supplied assistance.

It is also conceivable for system parameters that are at least partially mutually dependent or contradictory to be determined by means of an algorithm or on the basis of corresponding equations. The equations can be, in this context, those appended hereinafter.

It is furthermore conceivable for the system parameters to be proposed interactively in consideration of mutually exclusive properties or settings, and to be set after selection as well as (optionally after confirmation) automatically. These properties which mutually influence or indeed exclude one another are depicted in FIG. 1 as a "quality triangle." The factors that contradict or mutually exclude one another are the maximum resolution, maximum brightness, and maximum imaging speed.

In particularly advantageous fashion, the system parameters are retrieved, in consideration of the definitions, from an expert system stored in the database, said expert system possibly comprising on the one hand empirical values and on the other hand algorithms in consideration of the quality triangle. It is furthermore conceivable for the system parameters to be ascertained, in consideration of the definitions, using fuzzy logic, and to be set after selection or automatically (optionally after confirmation by the user), actions in accordance with fuzzy logic possibly being incorporated into the expert system.

The user interface could furthermore be designed in such a way that upon definition and/or modification of at least one system parameter, a report is interactively given that (and, if affirmed, to what extent) image acquisition will be influenced in terms of an image acquisition property, for example in terms of resolution, sampling, etc. This is therefore a "far-sighted" user interface system, specifically one in which effects in terms of quality are reported. It is furthermore advantageous if there is conveyed to the user, before, during, and after image acquisition, a datum regarding the image acquisition quality that is to be obtained, specifically so that a check can be made as to whether the image quality is sufficient based on the one hand on the selected system parameters and on the other hand on those defined by the system.

The imaging parameters or system parameters set or modified by the user could be analyzed by the imaging program. If one or more system parameters was "incorrectly" set by the user, the program can, automatically and on its own initiative, interactively guide the user with the goal of reestablishing an optimum system setting. For this purpose, a "quality demon module" of the program would need to analyze the present system parameters continuously during use, and automatically call the interactive module if a non-optimal setting is present.

Lastly and in very particularly advantageous fashion, in particular in order to introduce new users, the user interface could interactively execute a teaching program in which the user is instructed as to optimal imaging strategies, specifically on the basis of specimen-specific or problem-specific system settings. The interactively executed teaching program could also be designed as a training program for already-experienced users, specifically in order to be able to improve the quality of the work done by users working with the fluorescence microscope.

As shown in FIG. 1, three features in confocal scanning laser scanning microscopy are mutually exclusive, namely maximum imaging speed, maximum

resolution, and maximum brightness. For example, if a user wishes to achieve high spatial resolution, a long scan is necessary. The signal thereby obtained is usually greatly reduced. The situation is the same with the other variables that are depicted in FIG. 1 in the context of the "quality triangle" therein.

In confocal laser scanning microscopy, the user of a corresponding apparatus is confronted essentially with the following problems:

- with fluorescent specimens, the sample bleaches excessively;
- the image data are "noisy";
- the measurement time is too long; and
- the resolution of the image is too low.

Application of the method according to the present invention makes available to the user a user interface or computer interaction system in which an optimum system setting is possible depending on the specific application, and corresponding imaging strategies are proposed and can be selected by the user. In all cases, the "quality triangle" shown in FIG. 1 can be utilized during optimization.

With the method according to the present invention, the user is furthermore offered assistance which allows him or her to obtain proposed solutions, in the form of a computer-assisted user interface, to every conceivable problem, in particular to the four aforementioned most common problems, additionally and specifically on the basis of selectable default settings. If an expert system is incorporated into the user interface, the user can draw upon empirical values from a wide range of microscopy applications and questions. In this context it is once again conceivable to implement a "teachable" system, i.e. a file that expands with every image that is acquired (automatically or after explicit confirmation by the user) and is evaluated positively. Methods such as "fuzzy logic" can be implemented, allowing the system settings to be further optimized.

Regarding the execution of the method and an optimization taking place therein, the reader is referred to FIG. 2 in reference to what is discussed above.

To elucidate the teaching that is claimed, a concrete exemplary embodiment will be explained below. The discussion thereof will be limited to the setting possibilities available with known systems. The specimen used is a Drosophila embryo stained with fluorescein. Its dimensions are approx. 200 μm laterally and approx. 100 μm axially. The maximum density ρ of the dye molecules and the properties of the dye used - such as the bleaching rate Λ, lifetime of the excited singlet or triplet state τ_s or τ_t, probability W_T of transition into the triplet state, effective cross section σ, and emission and excitation wavelengths λ_{Em} and λ_{Ex} - are known with sufficient accuracy:

λ_{Em}	=	520 nm	λ_{Ex}	=	490 nm
τ_t	=	10^{-6} s	τ_s	=	$4.5 \cdot 10^{-9}$ s
Λ	=	$3 \cdot 10^{-5}$	W_T	=	0.03
ρ	=	200 μm^{-3}	σ	=	$3.06 \cdot 10^{-16}$ cm^2

In order to ensure optimum adaptation of the system to the specimen being examined and to the user's requirements, the following process steps are performed:

1. Selection of the objective to be used. This follows directly from the dimensions of the specimen being examined. The minimum lateral resolution yields the number of pixels per image plane that is to be set.
2. Determination, from the imaging time and the properties of the fluorescent molecules, of the optimum irradiation intensity at the focus.
3. Calculation, from the focus area and the optimum irradiation intensity at the focus, of the laser output to be set.
4. Determination of the detection pinhole radius, as a compromise between the expected resolution and the expected signal-to-noise

ratio in the image. The number of optical sections follows from the axial resolution.

5. Adaptation of the photomultiplier voltage to the expected signal-to-noise ratio in the image.

For the specific example, the following data are obtained:

1. The maximum linear magnification of the objective should not exceed 50 (10 mm / 200 μm), and its working distance should be on the same order as the specimen thickness (0.1 mm). Given these boundary conditions, the numerical aperture (NA) should be maximized. This yields the following objective:

$$M = 40; \qquad WD = 80\ \mu m; \quad NA = 1.0.$$

With the objective selected, and in consideration of the indicated emission wavelength of the fluorescent molecules, the lateral resolution of the microscope is between 210 nm and 320 nm. The exact value depends on the pinhole radius that is set. Based on the Nyquist theorem, the number of pixels n_l to be imaged per line is

$$n_l = \frac{2}{r_{res}} \cdot 200\ [mm$$

and is therefore between 1920 and 1260. With the system being used, the number of pixels in the image can be set to the values 256^2, 512^2, or 1024^2. In the present example, the maximum pixel count of 1024^2 should be selected. Since this number is less than the values calculated with the Nyquist equation, it may happen that artifacts occur in the image. If so, either the scanned field must be made smaller by zooming, or an objective with a smaller numerical aperture must be used.

2. The imaging time per pixel depends on the imaging time per image plane and the number of pixels per image plane. Note that because the scanning mirror resets after each image line, only approximately half the imaging time

is used for detection. With an imaging time per image plane of approx. 2 seconds, the imaging time per pixel is approx. 1 μs; the irradiation time per pixel that is critical in terms of bleaching is approx. 2 μs.

The optimum irradiation intensity may be calculated as follows:

$$E_S = \frac{1}{\sigma \cdot \tau_S} \approx 7.3 \cdot 10^{23} \quad \frac{photons}{cm^2 \cdot s}$$

or

$$E_T = \frac{1}{\sigma \cdot \left(\tau_S + W_T \cdot \tau_T\right)} \approx 9.5 \cdot 10^{22} \quad \frac{photons}{cm^2 \cdot s}$$

If triplet states can be ignored, what results in this specific example is an irradiation intensity E_S that is eight times greater. This simplification is generally justified whenever the detection time per pixel is less than the lifetime of the triplet state. If the detection time is on the same order as the lifetime of the triplet state, as in the present example, its existence should be taken into account when calculating the optimum irradiation intensity.

Note that both values are based on the understanding that the best signal-to-noise ratio in the image is obtained if the emission rate of the fluorescent molecules is equal to their excitation rate. In principle, the system exponentially approaches this equilibrium. The calculation of E_S ignores the existence of the triplet states and assumes an equilibrium between the excited singlet state and ground state. The calculation of E_T assumes that equilibrium among all three states is immediately achieved. To obtain an equilibrium between the excitation rate and emission rate during the entire irradiation time, the irradiation intensity would need to attempt to go exponentially from E_S to E_T during that time. In each case, therefore, the two values represent a more less good approximation.

3. At the wavelength of 488 nm that is used, the photons have an energy of approx. $4 \cdot 10^{-19}$ J. The focal area $F_{focus} = \pi \cdot r_{Airy}^2$ is approx. $2.8 \cdot 10^{-13}$ m^2. This yields a radiation flux Φ of

$$\Phi = h\nu \cdot E_T \cdot F_{focus} \quad 100\ \mu W.$$

Since only approx. 10% of the coupled-in radiation flux arrives at the focus of the objective, the laser output should be set at approx. 1 mW. If triplet states can be ignored (i.e. with shorter imaging times), the laser output setting for fluorescein turns out to be approx. 8 mW.

4. The number of optical sections required is obtained from the thickness of the specimen, the axial resolution capability, and the Nyquist theorem. Since a detection pinhole setting greater than 8 optical units results in poor image quality, the axial resolution is between 0.6 μm and 1.8 μm. The number of optical sections, assuming utilization of the entire working distance, is therefore between 270 and 90. Since only a single channel is being used for detection, the size of the acquired data set is therefore between 270 and 90 megabytes. At this point the number of optical sections may also be limited in practical terms by the system's working memory.

In general, the user will strive for maximum axial resolution, in which context the signal-to-noise ratio should not fall below a specific threshold.

With a detection pinhole radius set at approx. 2 optical units, the microscope achieves almost its maximum axial resolution capability. The lateral resolution is approx. 260 nm.

The number n_z of sections to be imaged is obtained from the axial resolution z_{res}, the thickness of the specimen, and the Nyquist theorem:

$$n_z = \frac{2}{z_{res}} \cdot 80mm = 270$$

From the axial resolution and the lateral resolution, assuming an ellipsoidal focus, we obtain the detected pixel volume V_{pixel}:

$$V_{pixel} = \frac{4}{3} \cdot \pi \cdot z_{res} \cdot r_{res}^2 \approx 0.17 mm^{-3}$$

The maximum density ρ of the fluorescent molecules is 200 molecules per μm^3. The number of molecules N_{focus} in the focus is therefore:

$$N_{focus} = \rho \cdot V_{pixel} \quad 34.$$

If the irradiation intensity is optimally set, the emission rate E_m is equal to the excitation rate. In other words, in dynamic equilibrium half the molecules are in the ground state and the other half are in the excited state. In the present example, triplet states cannot be ignored. The emission rate per molecule is therefore:

$$Em = \frac{1}{2 \cdot \left(\tau_S + W_T \cdot \tau_T\right)} \approx 15\, ms^{-1}$$

For a detection time of 1 μs, approx. 510 photons are therefore emitted per pixel. Since the system, with the objective being used, detects only approx. 1% of all emitted photons, the value obtained for the detected signal level is approx. 5 photons per pixel. This corresponds to a signal-to-noise ratio (S/N) of approx.:

S/N 2.3

If this value is below the threshold defined by the user, some resolution must be sacrificed. The result is to increase the detected pixel volume and thus the number of photons detected per pixel. The number of layers to be imaged also decreases, resulting in less bleaching of the specimen.

If resolution is not sacrificed in favor of a better signal-to-noise ratio, the number of optical sections remains 270. This means that the entire

specimen surface is exposed, 270 times for a period of 2 μs, to the irradiation intensity calculated above. The proportion n/n_0 of molecules that remain unbleached thereafter is:

$$\frac{n}{n_0} = \exp\left(-\Lambda \cdot \frac{270 \cdot 2\mu s}{2 \cdot (\tau_S + W_T \cdot \tau_T)}\right) \approx 0.8$$

Since a capability for increasing the imaging time for a greater number of image sections is not provided in the system used, and is technically difficult to implement, the result of bleaching of the specimen is that the brightness of the layers imaged last is approx. 80% that of the layers imaged first. The signal-to-noise ratio is reduced to approx. 2.

5. If this signal-to-noise ratio is also accepted by the user, all that remains is to adapt the photomultiplier voltage to the maximum expected signal level of approx. 5 photons per pixel. For the indicated imaging time per pixel, the resulting photomultiplier voltage is approx. 720 V.

Note that the specimen being used has a relatively high density of fluorescent molecules. It may happen in practice that samples having fewer than 10 molecules per μm^3 are examined. In such cases, averaging must be performed over several measurements in order to achieve adequate image quality.

The lifetime of the triplet state can be reduced by the presence of, for example, oxygen; or in many dyes, even in the isolated state, it is shorter than that of the fluorescein triplet state. In the present example, a decrease in the lifetime of the triplet states results in an increase in the optimum irradiation intensity, higher emission rates, and therefore also a better signal-to-noise ratio in the image. Because of the greater radiation load, however, the specimen is more severely bleached during imaging.

The spectrum of bleaching rates in the various dyes is very broad (depending on the molecules' environment), and in solid preparations can be reduced a

great deal using anti-bleaching agents. The bleaching rate of fluorescein represents a typical average value.

In conclusion, be it noted very particularly that the method steps and exemplary embodiments recited above represent preferred embodiments, but do not limit the teaching of the invention to those embodiments of the method that is claimed. All the method steps can also be applied, with reference to the teaching claimed in accordance with Claim 1, in isolated form and thus independently of the other Claims.